

Effects of Triazolam Microinjections into the Peri-fornicular Region on Sleep in Rats

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The hypocretin/orexin (Hcrt/OX) receptor-ligand system is involved in the pathophysiology of narcolepsy/cataplexy and may play a role in the physiologic regulation of sleep and waking. Most Hcrt/OX neurons are located in the peri-fornicular region (PeF) of the posterior hypothalamus. In this study, we explored the possibility that some pharmacological effects on sleep may also be mediated by this system, by microinjecting the clinically used benzodiazepine hypnotic triazolam (TR) into the PeF of rats. Fourteen rats received bilateral microinjections of vehicle and TR (0.25ug and 0.50 ug) into the PeF, in randomized order, followed by 4 hours of sleep/wake recordings. Data were analyzed by repeated measures analysis of variance for dose (vehicle, TR 0.25ug and 0.50ug) and time (hours 1-4) factors. In five additional rats, TR (0.50 ug) was administered in combination with the Type A γ -aminobutyric acid-benzodiazepine receptor (GABA_A-BZD) antagonist, flumazenil (0.95 ug). TR (0.25 and 0.50ug) significantly decreased wake and intermittent wake time and increased non-rapid eye movement (NREM) and total sleep times in the two hours following injections. TR (0.25 and 0.50ug) decreased NREM sleep latency and TR (0.25ug) decreased rapid-eye movement latency. The effects of TR (0.50 ug) were blocked by flumazenil (0.95 ug). TR significantly enhanced sleep when microinjected into the PeF region. These data provide a basis for the hypothesis that the function of the Hcrt/OX system may be altered by exogenously administered benzodiazepines, and potentially by endogenous ligands of the GABA_A-BZD receptor. **(Sleep and Hypnosis 2003;5(3):154-162)**

Key words: triazolam, flumazenil, peri-fornicular area, sleep, hypothalamus

INTRODUCTION

The hypothalamus plays a major integrative role in the homeostasis of a variety of

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Acknowledgement: This study was partially supported by NIH grants K07 HL03640 and R01 DA10682 to W.B.M.

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Accepted June 2, 2003

physiological processes, including sleep and waking. The preoptic area of the anterior hypothalamus (POAH) and the tuberomammillary nuclei (TMN) of the posterior hypothalamus (PH) interact to form part of an intrahypothalamic sleep/wake control system (1-6). For example, γ -aminobutyric acid (GABA)-containing neurons from the POAH project to and provide inhibitory inputs to histaminergic neurons in the TMN (4,7), while the latter sends histaminergic afferents to

the POAH (8,9). Muscimol, a GABA_A receptor agonist, injected into the PH region including the TMN, enhances sleep, whereas histamine (HA) and HA1 receptor agonists injected into the medial preoptic area (mPOA) promote wakefulness (1,2). Therefore, many studies provide evidence that interactions between structures in the POAH and PH represent one important mechanism for the alternation of sleep and wake states.

Alteration in the ligand-receptor system for hypocretin/orexin (Hcrt/OX), a neuropeptide and putative neurotransmitter synthesized in the PeF region of the PH [10-13], was implicated in the pathophysiology of narcolepsy/cataplexy (14-16). Hcrt/OX immunoreactivity in the POAH, as well as other sleep/wake-related areas of the forebrain and brainstem, suggests that it may also be involved in arousal state regulation in normal (non-narcoleptic) animals (12,17). Consistent with this anatomy, intracerebroventricular (ICV), mPOA, basal forebrain (BF) and TMN injections of Hcrt1/OXA have been followed by increased wakefulness and locomotor activity, decreased deep non-rapid eye movement (NREM) and rapid eye movement (REM) sleep and prolonged REM latency in rats [18-23]. Hcrt/OX modulates in vitro activity of neurons in the locus coeruleus (LC) and laterodorsal tegmental nucleus (24-26), areas densely to moderately innervated by Hcrt/OX (12). In combination, these studies show involvement of the Hcrt/OX receptor-ligand system in the pathophysiology of narcolepsy/cataplexy as well as the regulation of normal sleep and wake states.

The PeF falls in the projection pathway of histaminergic neurons from the TMN and GABAergic projections from the POAH, although the potential function of these afferent inputs has not been examined (7-9). The PeF may serve an important role in hypothalamic sleep-wake regulation, in part through reciprocal interactions with the TMN and POAH. For example, Hcrt1/OXA-and

Hcrt2/OXB depolarize and excite TMN neurons (27,28), the majority of which contain receptors for these ligands (28). We have found at the behavioral level that microinjections of HA into the PeF significantly induce EEG arousal and attenuate REM sleep in rats (Laposky and Mendelson, in preparation). In this study, we investigated the specific hypothesis that triazolam (TR) microinjections into the PeF of rats would attenuate wakefulness and enhance NREM and REM sleep compared to vehicle injections and that these effects would be blocked by flumazenil (FLU), a GABA_A-BZD receptor antagonist.

METHODS

Fourteen male 250-300 gm Sprague-Dawley rats (Harlan Co., Indianapolis, Ind.) received bilateral microinjections of TR (0.25ug and 0.50ug/.4ul vehicle) and vehicle (VEH) into the PeF in randomized trials separated by 3 days each. Five other rats received bilateral microinjections of VEH-VEH, FLU (0.95ug)-VEH (0.95ug), VEH-TR (0.50ug), and FLU (0.95ug)-TR (0.50ug) into the PeF in randomized trials separated by 3 days each. This dose of FLU (0.95) has been previously shown to block sleep-inducing effects of TR and ethanol in the mPOA of rats (29-31). Experimental procedures followed a protocol approved by the Institutional Animal Care and Use Committee and were in accord to the policy of the American Physiological Society.

Surgical Procedure

Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (70mg/kg) and xylazine (6mg/kg) and then placed in a stereotaxic apparatus with the incisor bar set at +3.0 mm. Following a scalp incision, the skull was exposed, cauterized to stop bleeding and cleaned for identification of bregma and lambda sutures. Bilateral stainless steel screws were implanted into the skull 5

mm lateral to the central suture and 1 mm anterior to bregma and lambda, respectively, to record the electrocorticogram (ECoG). Two other pieces of this wire were stripped at the end, and inserted bilaterally into the neck musculature to record electromyogram (EMG) activity. These wires were connected to an Amphenol socket by short lengths of Teflon-coated stainless steel wire.

The stereotaxic coordinates for PeF guide cannulae were: A-P -3.14 , M-L ± 1.2 , D-V -7.4 , according to Paxinos and Watson (32). Holes were drilled at these locations, the dura gently disrupted and bilateral 24-gauge stainless steel guide cannulae lowered to 1.0 mm above the PeF. Before the rat was released from the stereotaxic apparatus, the entire assembly of cannulae, electrodes and Amphenol socket was stabilized with dental acrylic. The incision area was sutured around the assembly and treated with triple antibiotic containing bacitracin, polymycin and neomycin. Finally, 31-gauge stainless steel blocking stylets were inserted into the guide cannulae and a protective plug was placed in the Amphenol socket.

The rats were given at least one week to recover from surgery. They were housed individually in smooth-walled plastic cages with a 12:12 light:dark cycle (lights on at 8:00 AM) and an environmental temperature of 25 C°. These were the same environmental conditions in which the rats were housed prior to the experiment. In order to adapt the animals to the recording environment, their regular housing cages were placed in the recording chambers at 4:00 PM on the day before the experiment. At 9 AM the following day, rats were attached to a flexible recording cable for 1 hour of adaptation to the cable. Microinjections were performed at 10:00 AM.

Microinjections procedure

A 31-gauge stainless steel injection stylet was connected to a microliter Hamilton syringe by a

short length of polyethylene (PE 20) tubing. The injection stylet was 1.0 mm longer than the guide cannulae, so that when inserted, it would extend into the precise area of brain tissue. Prior to injection, vehicle, TR and FLU were warmed to 37.0 C° and drawn into the Hamilton syringe. After removing the blocking stylet from each guide cannulae, the injection stylet was inserted and vehicle/drug was delivered over the period of one minute and left in place for an additional 30 seconds. A small air bubble, previously drawn into the PE20 tubing, was used to verify accurate drug delivery. During injections, rats were wrapped lightly in a towel, which prevented the need for hand-restraint. The total injection time was 4-5 minutes per animal. Volumes and infusion rate were derived from the study by Myers (33), to minimize tissue damage and to restrict diffusion of drug from the injection site. TR (Sigma) and FLU (vondor) were dissolved in a 1:1 solution of emulphor:ethanol and diluted with 9 parts artificial cerebrospinal fluid (0.5mM NaH₂PO₄, 0.25mM Na₂HPO₄, 0.4mM MgCl₂, 0.65mM CaCl₂, 3mM KCL, 128mM NaCl, 25mM NaHCO₃, and 250mg/l bovine serum albumin, pH=7.4) to give final concentrations of TR (0.25ug and 0.50ug) and FLU (0.95ug) per 0.4ul of solution. Bilateral microinjections were made in the PeF using 0.20ul vehicle, TR or FLU on each side, for a total volume of 0.40ul per animal.

Data collection and analysis

Following the injection, each rat was returned to the recording chamber and data was collected using a Grass Model 78 polygraph. Three channels representing bifrontal ECoG (sensitivity [S]=20, low-frequency filter [LF]=.3, high-frequency filter [HF]=60), fronto-occipital ECoG (S=20, LF=.3, HF=60) and EMG (S=20, LF=10, HF=90) were recorded for 4 hours for each rat. The paper speed was 10 mm/sec and the oscillograph was calibrated at 5 uv/mm. Following the recording,

animals were returned to the housing facility and two days later were run in the second injection condition.

At the end of the study, a single investigator (who was blind to the treatment condition) classified each 30 second epoch as “waking”, “non-REM”, or “REM” sleep. Sleep-wake data was reported as measures of sleep latency (time from microinjection until the first three consecutive 30 second epochs of sleep), intermittent wake time (waking time after initial sleep onset), REM latency (time from sleep onset until the first 2 consecutive epochs of REM sleep) and minutes of wake, NREM, REM and total sleep time. Data was analyzed using a repeated measures analysis of variance (ANOVA) for injection trial (vehicle, TR, FLU) and time (hours 1-4) factors. Significant interactions were followed up using Scheffe post-hoc tests, as described by Kirk (34).

Histology

Histological examination of injection sites was performed by anesthetizing each animal with ketamine (70mg/kg) and xylazine (6mg/kg) and perfusing transcardially with rinse (0.9% saline and heparin) and formalin (10%). The rat was decapitated, and the brain was removed and stored in a formalin (10%) solution. Coronal brain sections (45-50 μ m) were cut on a freezing microtome and stained with cresyl violet. The tip of the injection cannula track was then determined by light microscopy.

RESULTS

Microinjection sites

In 9 out of 14 rats, microinjections were accurately placed in the PeF region, as shown schematically in Figure 3. In a group of 5 rats, injections were placed outside of the PeF region (Figure 3), corresponding to the ventromedial,

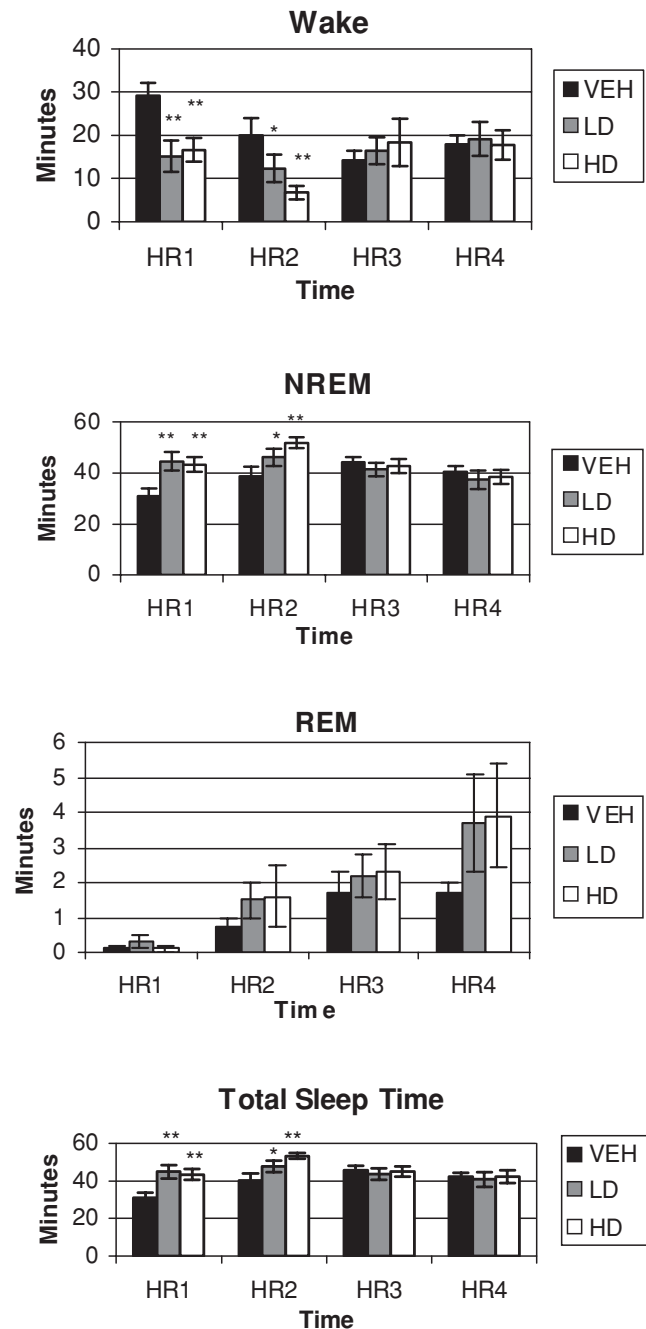


Figure 1. Sleep measures for sequential hours 1-4 following injection of vehicle (Veh), triazolam 0.25 ug (LD) and 0.5 ug (HD).

ventrolateral, dorsomedial and dorsal hypothalamic areas (32). In a separate group of animals, injection cannulae were accurately placed in 4 out of 5 rats that received a combination of FLU (0.95ug) and TR (0.50ug),

similar to the sites shown in Figure 1.

Wake, NREM, REM and TST

A within-subjects ANOVA for dose (vehicle, TR 0.25ug and 0.50ug) and time (hours 1-4) factors revealed significant interactions for minutes of wake ($F=2.42$ (2,18), $p<0.03$), NREM ($F=3.05$ (2,18), $p<0.01$) and TST ($F=2.6$ (2,18), $p<0.03$). Scheffe post-hoc tests were performed to compare doses independently at each hour (1-4), as detailed in Figure 3. Wake time was decreased by TR (0.25ug) in hour 1 ($p<0.01$) and hour 2 ($p<0.05$), whereas NREM ($p<0.01$ and $p<0.05$) and TST ($p<0.01$ and $p<0.05$) were increased in the first two hours, respectively. TR (0.50ug) had similar effects for wake, NREM and TST in the first 2 hours (all comparisons significant at $p<0.01$).

Sleep latencies and intermittent wake time

Separate one-way ANOVAs showed significant dose (VEH, TR 0.25ug and 0.50ug) main effects for NREM sleep latency ($F=10.24$ (2,18), $p<0.001$), REM latency ($F=4.90$ (2,18), $p<0.05$) and intermittent wake time ($F=9.89$ (2,18), $p<0.001$), as detailed in Figure 3. Post-hoc comparisons between dose and VEH showed significantly reduced NREM latency by TR 0.25ug ($p<0.01$) and TR 0.50 ($p<0.01$). REM latency was decreased by TR 0.25ug ($p<0.05$). Intermittent wake time decreased with TR 0.25ug ($p<0.01$) and 0.50ug ($p<0.01$) injections.

Injections outside of the PeF region

In 5 rats, injections were made in areas surrounding the PeF (Figure 1). There were no apparent differences in sleep parameters between VEH and TR (0.25ug and 0.50ug) trials in these animals (data not shown). Because of the small sample size, ANOVA was not used to analyze these data.

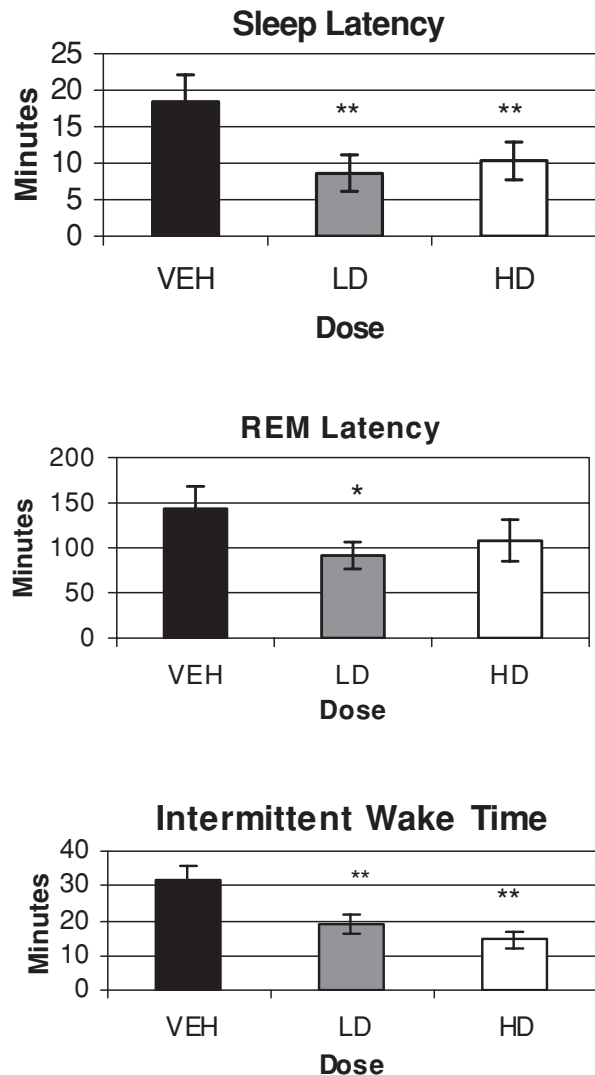


Figure 2. Sleep latency, REM latency and intermittent waking time (waking time after initial sleep onset) following injection of vehicle (Veh), and triazolam 0.25 ug (LD) and ug (HD).

Combination of flumazenil and triazolam

Preliminary data were collected in 5 rats to test whether FLU (0.95ug) would block the effects of TR (0.50ug) in the PeF. This sample size was not amenable to proper ANOVA testing, and non-parametric equivalents to factorial designs are not available, therefore, descriptive data only are presented in Table 1. Visual inspection showed that VEH-TR (0.50ug) injections clearly increased NREM and TST and decreased wake time, intermittent wakefulness and NREM sleep latency within

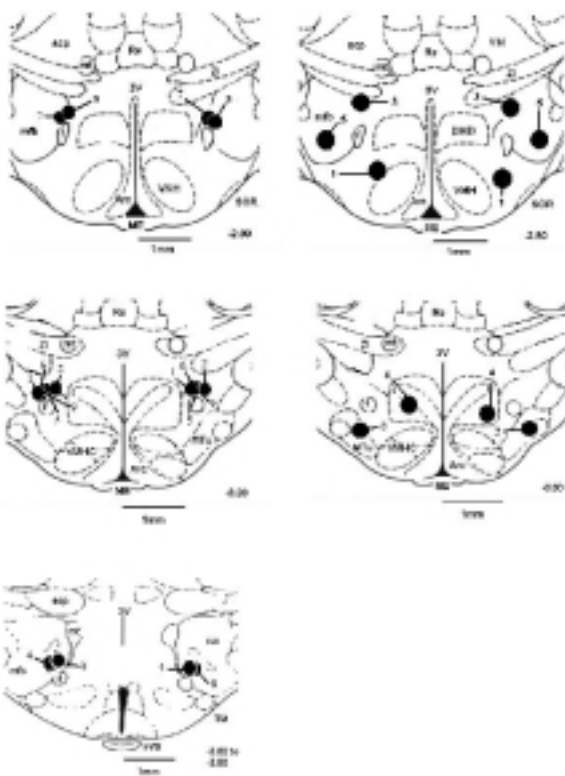


Figure 3. Schematic representation of histologically identified bilateral microinjection sites in the peri-fornicular area for each animal. Arc = acruate nucleus; f = fornix; InfS = infundibular stalk; LH = lateral hypothalamus; MTu = medial tuberomammillary nucleus; ME = median eminence; mfb = medial forebrain bundle; mt = mammillothalamic tract; 3V = third ventricle; VMH = ventromedial hypothalamic nucleus

the 2 hours following injections, replicating the data in the initial group of 9 rats (presented above). FLU (0.95ug) blocked the effects of TR (0.50ug), in that values (Table 1) from FLU(0.95ug)-TR(0.50ug) injections were similar to VEH-VEH and VEH-FLU (0.95ug) trials.

DISCUSSION

In this study, bilateral microinjections of TR, a GABA_A-BZD receptor agonist, into the PeF area significantly decreased wakefulness, enhanced NREM sleep, and reduced NREM and REM latencies in rats. These effects were blocked when the GABA_A-BZD receptor antagonist, flumazenil was co-administered into the PeF. These data suggest that endogenous GABA may represent an important afferent signal to PeF neurons, and that Hcrt/OX tone may be modulated, in part, by GABAergic input.

The PeF region contains the major population of Hcrt/OX-containing neurons, and Hcrt/OX immunoreactivity has been identified in many important sleep-related regions of the forebrain and brainstem (12).

Table 1. Sleep/wake values (minutes +/- S.E.) following triazolam and flumazenil microinjections into the peri-fornicular (PeF) area

	Wake	HS	PS	TST	SL	RL	IWT
VEH-VEH	32.5(3.8)	27.0(3.7)	0.5(0.2)	27.5	19.3 (2.9)	158.8(29.2)	36.6 (4.5)
2	23.4(4.2)	35.4(3.8)	1.2(0.4)	36.6	--	--	--
3	16.7(2.7)	40.5(3.0)	2.8(0.8)	43.3	--	--	--
4	18.9(3.5)	38.5(3.8)	2.2(0.9)	40.7	--	--	--
VEH-TR	15.7(2.9)	44.1(3.0)	0.2(0.1)	44.3	11.7(3.6)	142.7(22.9)	13.1(2.5)
2	9.1(2.9)	49.4(2.7)	1.5(0.5)	50.9	--	--	--
3	16.5(3.2)	40.8(3.0)	2.7(1.1)	43.5	--	--	--
4	20.3(3.8)	36.6(4.2)	3.2(0.9)	39.8	--	--	--
FLU-VEH	30.7(2.4)	29.2(2.3)	0.1(0.1)	29.3	23.6(2.9)	161.1(27.1)	28.0(2.8)
2	20.9(3.1)	38.3(3.2)	0.8(0.2)	39.1	--	--	--
3	15.5(3.6)	43.2(3.3)	1.3(0.7)	44.4	--	--	--
4	16.8(4.1)	40.1(3.7)	3.1(1.1)	43.2	--	--	--
FLU-TR	29.7 (4.1)	29.6(3.9)	0.7(0.2)	30.3	21.8(3.7)	169.8 (33.6)	30.1(3.8)
2	22.2(3.3)	36.8(3.7)	1.0(0.2)	37.8	--	--	--
3	17.4(4.6)	40.2(4.1)	2.4(1.0)	42.6	--	--	--
4	15.6(4.1)	40.6(3.7)	3.8(1.4)	44.4	--	--	--

W=wake; HS=high voltage sleep; REM=rapid eye movement sleep; TST=total sleep time; SL=Sleep latency; RL=REM latency; IWT = intermittent wake time; VEH=vehicle; TR=triazolam (0.50ug); FLU=flumazenil (0.95ug).

The deletion of Hcrt/OX or mutations of Hcrt/OX receptors have been implicated in the pathophysiology of narcolepsy/cataplexy, a sleep disorder characterized by excessive sleepiness and abnormally enhanced indices of REM sleep (14-16). With regard to normal physiologic sleep, ICV and mPOA microinjections of Hcrt1/OXA have wake promoting and NREM/REM suppressing effects in rats (18-20,22). Administration of Hcrt1/OXA by microdialysis into the BF (diagonal band of Broca) and TMN significantly decreased REM and slow wave sleep (21,23). Infusions of Hcrt1 and 2 into the medial septum and substantia innominata of sleeping rats decreased wake onset latency and increased the amount of wakefulness following injection (22). Hcrt1/OXA microinjections into the LC of rats decreased REM sleep (24) and in the LDT of cats increased wakefulness and reduced REM sleep (35). Therefore, Hcrt/OX has *in vivo* pharmacologic activity in known sleep-related regions of the forebrain and brainstem, and may also function as an endogenous substance involved in maintaining normal amounts of sleep and wakefulness.

While Hcrt/OX immunoreactivity and effects of localized Hcrt/OX injections have been studied, there are essentially no data regarding the afferent systems modulating PeF neurons. The PeF falls within the pathway of GABAergic POAH and histaminergic TMN neurons, and may form part of an intra-hypothalamic sleep-wake control system, which has previously been proposed for interactions between the POAH and TMN (1,4,5,7,9,23,36). Results from this study indicate that TR, an allosteric agonist of the GABA_A-BZD receptor complex, modulates activity in the PeF region, as evidenced by changes in sleep/wake states in the two hours following injections. In combination with previous injection studies showing that activation of the Hcrt/OX receptor-ligand system consistently increases wakefulness, we hypothesize that TR exerted inhibitory properties on Hcrt/OX-containing

neurons, and decreased Hcrt/OX activity at efferent forebrain and brainstem target sites, therefore promoting sleep. The effects of TR were blocked by co-administration of flumazenil, indicating involvement of the GABA_A-BZD receptor/inhibitory ionotropic chloride channel, in sleep-related mechanisms in the PeF.

The expression of c-fos in PeF neurons, including those containing Hcrt/OX, was positively correlated with physiological wake time, as well as arousal following methamphetamine administration (37,38). Furthermore, a certain proportion of PeF neurons have wake-related firing patterns (39). These data provide indirect support for the hypothesis that inhibition of Hcrt/OX neurons in the PeF may lead to increases in sleep. Because TR may bind to any GABA_A-BZD receptor in the injection area, the degree to which TR and/or GABA directly modulates Hcrt/OX-containing neurons may be established in future experiments. There are various neurotransmitters represented in the PeF, such as high concentrations of melanin corticotrophic hormone and histaminergic fibers from TMN projections. Therefore, indirect effects of TR through these neurochemical systems in the PeF cannot be ruled out.

Alternatively, TR may have exerted indirect effects on sleep through the integration or independent regulation of other physiological functions, such as thermoregulation or metabolic balance (12). Temperature, and sleep are closely linked in normal physiological conditions, and neurons responsive to both changes in sleep-wake state and temperature exist throughout the POAH (40). We did not measure brain or body temperature in this study and future investigations may clarify whether the PeF is involved in thermoregulation, particularly in light its involvement with metabolic (feeding) and autonomic (cardiorespiratory) control (12).

Even though TR microinjections were made

directly into the PeF, it is possible that some of the substance diffused to include outlying histaminergic neurons in the TMN. Microinjections of muscimol (41) and histamine (42) into the PH, including the TMN region enhance sleep and wake, respectively. The extent to which Hcrt/OX and histaminergic neurons overlap in the PH may be established in future studies. Some localization of effect was confirmed, however, in that TR microinjections approximately 1 mm outside of the PeF, in each

direction, failed to produce hypnotic effects, indicating that the functional actions of TR did not spread beyond a 1 mm injection radius.

In summary, the POAH and TMN have been studied as a potential intra-hypothalamic reciprocal sleep-wake control system. Our results indicate that the PeF region is responsive to pharmacologic input of a GABA_A-BZD receptor agonist, and may represent another hypothalamic area interacting with the POAH and TMN.

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